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New Small Polypeptides Associated with DNA-Dependent RNA Polymerase of *Escherichia coli* after Infection with Bacteriophage T4

(polyacrylamide gel electrophoresis/maturation-defective mutants/gene 55)

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ABSTRACT **Four new small polypeptides are associated with DNA-dependent RNA polymerase from *E. coli* after infection with T4 phage. The new polypeptides are easily detected in RNA polymerase from *E. coli* cells labeled with amino acids after phage infection. Their molecular weights range from 10,000 to 22,000, as detected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. All four polypeptides are found after infection with either wild-type T4 phage or T4 early amber mutants in genes 44, 42, 47, and 46. None of the polypeptides is labeled significantly before 5 min after infection at 30°. When two maturation-defective amber mutants in gene 55 of T4 phage are used for infection, a polypeptide with a molecular weight of 22,000 is absent. When a maturation-defective amber mutant in gene 33 of T4 phage is used, another small protein is absent.**

In a previous report (1) I described a double-labeling technique, designed to detect changes in the DNA-dependent RNA polymerase of *Escherichia coli* (EC 2.7.7.6) after infection with T4 phage. I reported that when *E. coli* cells are labeled with [^{3}H]aminoacids before infection and with [^{14}C]-aminoacids after infection with T4 phage, the core RNA polymerase isolated from the cells contains both isotopes. ^{3}H was found in the β, β', and α subunits of the enzyme, while ^{14}C was found in a component with a molecular weight of about 10,000, as analyzed by Sephadex gel filtration in the presence of sodium dodecyl sulfate (SDS). The present paper shows that the ^{14}C-labeled material consists of four small polypeptides when examined by polyacrylamide disc gel electrophoresis in the presence of SDS. The kinetics of their synthesis and their relationship to maturation-defective amber mutants in T4 genes 33 and 55 are described.

MATERIALS AND METHODS

Materials. The amber mutants—*am*BL292 (gene 55), *am*552 (gene 55), and *am*N134 (gene 33)—were gifts of Dr. E. P. Geiduschek. The double mutants—*am*N55-*am*B14 (genes 42 and 46) and *am*N55-*am*A456 (genes 42 and 47)—were gifts of Dr. John S. Wiberg. The amber mutant phage stocks were prepared with the su_I^+ host, *E. coli* CR63.

The labeled L-aminoacids were obtained from Schwarz BioResearch, Inc. and suitably diluted with the unlabeled materials. Incorporation of labeled amino acids into trichloroacetic acid-precipitable material was linear with time during the labeling periods.

Growth of E. coli Cells and Isolation of RNA Polymerase. For the labeling experiments, *E. coli* B was grown in M9 medium (2) supplemented with 0.2% glucose, tryptophan (50 μg/ml), 0.1 mM $CaCl_2$, and 3 μM $FeCl_3$. Small (100–400 ml) cultures were grown to a cell density of 5×10^8/ml and then infected with T4 phage at a multiplicity of 8, either directly or after dilution of the cells to a density of 3.3×10^8/ml, by addition of 0.5 volume of fresh M9 medium. At 2 min after infection, 0.1 ml of each culture was removed and diluted for plating of surviving cells. In all cases, survival was less than 1%. After the labeled cells were collected by centrifugation, they were washed with 20 ml of 10 mM Tris buffer (pH 7.8) containing 10 mM $MgCl_2$ and 20 mM NH_4Cl, and frozen. The frozen cells were mixed with 3 g of uninfected *E. coli* B (grown in the same way as above); RNA polymerase was purified as described (1). Instead of two sucrose gradients, two 12–30% glycerol gradients were used for band sedimentation of the enzyme. The first gradient solution contained 0.4 M KCl; the second gradient contained 0.05 M KCl. Uninfected, rather than infected, *E. coli* B cells were usually used as carrier, because with them the yield of enzyme was higher in the protamine sulfate step in the purification procedure.

For examination of the RNA polymerase from infected cells by protein staining of gels, 50 g of cells infected with T4 *am*N82 phage were prepared as described above. The cells were collected 20 min after infection at 37°. RNA polymerase was purified as described (1) except that four consecutive band sedimentations in 12–30% glycerol gradients were performed instead of two. The four centrifugations were necessary to achieve the desired state of purity. The first and third gradient solutions contained 0.4 M KCl and the second and fourth contained 0.05 M KCl. The enzyme was precipitated from the active fractions of each gradient with ammonium sulfate at 60% saturation.

Polyacrylamide Disc Gel Electrophoresis. The enzyme preparations (usually 20–60 μg in about 0.2 ml) were prepared for electrophoresis by dialysis for 14–24 hr against 10 mM sodium phosphate buffer, (pH 7.1), containing 10% glycerol-0.1% SDS-0.1% 2-mercaptoethanol. Electrophoresis was performed as described by Weber and Osborn (3); 12% polyacrylamide gels (6 cm long) with an acrylamide:bis-acrylamide ratio of 110 were generally used. Before electrophoresis, the 2-mercaptoethanol concentration was increased to 1%. The electrophoresis was performed at room temperature for 3–5 hr until the marker dye band (bromphenol blue) migrated about 5 cm. The gels were either stained for 2 hr with Coomassie blue (3) or prepared immediately for counting.

For counting, the gels were frozen and sliced into 1-mm pieces with a Mickle Laboratory gel-slicer. The slices were

Abbreviation: SDS, sodium dodecyl sulfate.

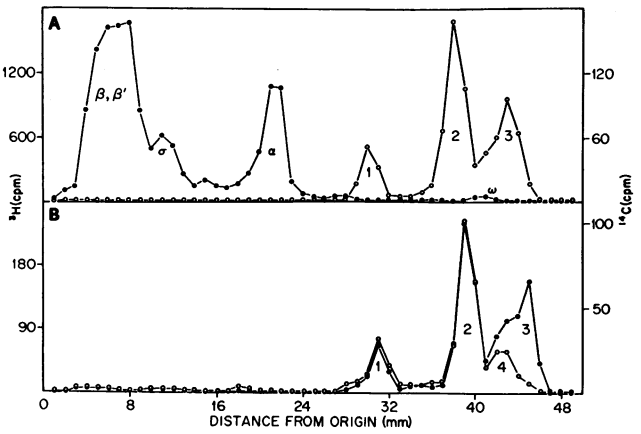

FIG. 1. Gel migration patterns of RNA polymerase from two labeling experiments with T4 *am*N82 phage. For Fig. 1*A*, a 200-ml culture of *E. coli* B, grown from a cell density of 1×10^8/ml to 5×10^8/ml, was labeled at 37° with [^{3}H]leucine (1 mCi, 2 mg). The cells were then removed from the medium by centrifugation and suspended in 400 ml of fresh M9 medium containing 10 mg of leucine. When the new culture reached a cell density of about 5×10^8/ml, it was infected by the addition of T4 *am*N82 phage at a multiplicity of 8. At 1.5 min after the addition of phage, 0.1 mCi of a [^{14}C]aminoacid hydrolysate containing 800 μg of each amino acid was added and, at 11 min after infection, the cells were poured into chilled centrifuge bottles and collected by centrifugation at 0°. The remainder of the procedure was as described in *Methods*. For Fig. 1*B*, a 100-ml culture of *E. coli* B was grown to a cell density of 5×10^8/ml at 37°. 50 ml of M9 medium was added and, after 10 min, T4 *am*N82 phage at a multiplicity of 8 was added. 1.5 min after addition of phage, a mixture of [^{3}H]leucine (1 mCi, 300 μg) and [^{14}C]proline (0.1 mCi, 250 μg) was added and the infected cells were collected by centrifugation at 11 min. ●——●, ^{3}H; ○——○, ^{14}C.

dried in scintillation vials, and then solubilized by incubation with 0.2 ml of 30% H_2O_2 for about 24 hr at 37°. 20 ml of scintillation fluid {containing 4 g of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene dissolved in a liter of toluene and methylcellosolve, mixed 2 to 1} was added to each vial. Radioactivity was measured with a Nuclear Chicago scintillation spectrometer.

RESULTS

Detection of new polypeptide bands

When *E. coli* cells were labeled with [^{3}H]aminoacids before infection with T4 *am*N82 phage and with [^{14}C]aminoacids after infection, the purified RNA polymerase contained both ^{3}H and ^{14}C-labels. The distribution of label in different subunits of the holoenzyme was determined after electrophoresis on a 12% polyacrylamide gel (Fig. 1*A*). Most of the ^{3}H-label was found in the four main subunits of the enzyme, i.e., β, β', σ, and α (4). The ratio of ^{3}H-label in the band of β, β' subunits to that in the band of the α subunits is about 4, as expected from their weight ratio (4). A less than stoichiometric amount of the σ subunit was recovered. The fastest-migrating small ^{3}H-band is in the position expected of the ω subunit of the enzyme (4). Three main ^{14}C-bands, labeled 1, 2, and 3 in Fig. 1*A*, were found on the 12% gels. Band 3 on many of the gels has a shoulder on the side (see Fig. 1*A*), suggesting the presence of a fourth band. In the experiment shown in Fig. 1*B*, [^{3}H]leucine and [^{14}C]proline were both used as labels after infection with T4 *am*N82 phage. [^{14}C]Proline was not significantly incorporated into the main band 3, but did label a minor band called 4. On the 12% gels, the minor band 4 was not separated well enough from band 3 to allow separate measurement, unless [^{14}C]proline was used as the label. The ω subunit of the normal enzyme is in a position identical to that of the minor band 4 on the 12% gels.

When phage-infected, rather than normal, cells were used as carrier, and the purification procedure was the same, a similar pattern of ^{14}C-labeling was observed. The patterns of ^{3}H-labeling differed in one respect—no σ subunit was recovered from the infected cells. The absence of the σ subunit in purified enzyme from infected cells was first reported by Bautz and Dunn (5). The presence of the ^{3}H-labeled σ subunit when uninfected cells are used as carrier must mean that σ is still present in infected cells and binds to normal core enzyme during the isolation procedure.

When a labeling experiment was performed as described in Fig. 1*A*, but with no phage added, the pattern of ^{14}C-label on the gels paralleled that of the ^{3}H-label.

The ^{14}C-bands 1 and 2 were found regardless of the purification step used. Band 3 was removed by phosphocellulose column chromatography and by extensive gradient centrifugation as described below. Oxidation of the enzyme with performic acid, as described for the RNA polymerase by Burgess (4), caused no change in the electrophoretic pattern of the ^{3}H- or ^{14}C-label.

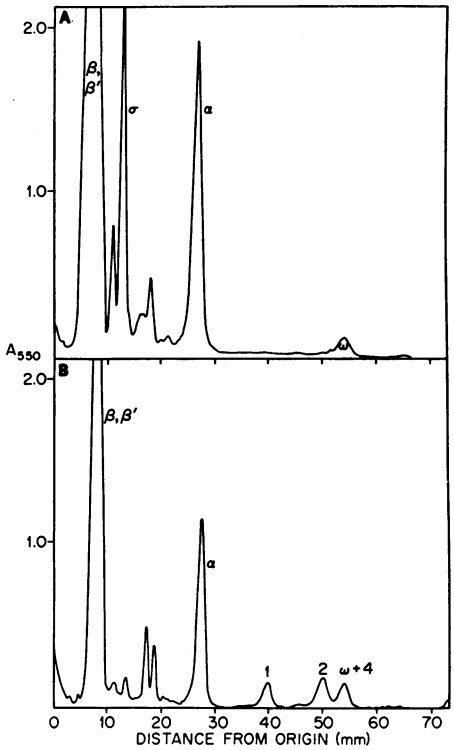

FIG. 2. Densitometer scans of stained gels of normal *E. coli* RNA polymerase (*A*, 30 μg) and of the enzyme from *E. coli* infected with T4 *am*N82 phage (*B*, 20 μg). The electrophoresis was on 12% gels as described in *Methods*. The gels were then stained for 2 hr with Coomassie blue and destained by soaking in several changes of a solution of 7.5% acetic acid–5% methanol. The gels were scanned at 550 nm.

Fig. 2 shows densitometer scans of stained gels of RNA polymerase from uninfected cells (Fig. 2A) and from cells infected with T4 *am*N82 phage (Fig. 2B). Both enzymes are 90-95% pure. As described above, the enzyme from infected cells lacks the σ subunit. Both enzymes have small amounts of high-molecular-weight impurities, which vary even from one centrifuged fraction to another. Two low-molecular-weight bands absent from the normal enzyme were found with the infected enzyme. They are in the positions of ^{14}C-bands 1 and 2 described above. The third small band on the gel of infected enzyme is in the position of the normal ω subunit and of ^{14}C-minor band 4, described above. No band corresponding to ^{14}C-band 3 was detected after the extensive gradient centrifugation used in the isolation procedure.

As determined by a polymerase assay (6), the enzyme from infected cells (Fig. 2B), without addition of the σ subunit, has a specific activity of 500-600 with calf thymus DNA as template and 60-100 with T4 DNA as template. Normal holoenzyme (Fig. 2A) has a specific activity of 1000 when calf thymus DNA is the template and the same or higher when T4 DNA is used. The activities found here for infected core enzyme are about the same as those reported (7).

Molecular weights of the new polypeptides

The sizes of the new small polypeptides were estimated by comparison of their mobilities on 12% gels with those of several standard proteins. Polypeptide 1 (band 1 in Fig. 1A) has a molecular weight of about 22,000; polypeptide 2 (band 2 in Fig. 1A), 14,000; polypeptide 3 (band 3 in Fig. 1A), 10,000; and polypeptide 4 (band 4 in Fig. 1B), 12,000.

The amounts of polypeptides 1 and 2 per RNA polymerase molecule were estimated by use of the approximate molecular weights of the new polypeptides and measurements of the peaks of the densitometer scans shown in Fig. 2B. The amount of polypeptide 1 is 0.5 molecule per molecule of polymerase, while the amount of polypeptide 2 is 1 molecule per molecule of polymerase. Labeling experiments in which the [^{3}H]leucine was introduced at the usual time before infection and then left in the medium throughout the infection period were also performed. By comparison of the amount of ^{3}H in the polypeptide bands with that in the α-subunit band, the following amounts of polypeptides per polymerase molecule were estimated: polypeptide 1, 0.2; polypeptide 2, 1.0; polypeptide 3, 0.9. Polypeptide 4, detected with [^{14}C]proline, has not been estimated. Why the two estimates of polypeptide 1 are so different is not known at this time.

Kinetics of synthesis of the new polypeptides

A study of the kinetics of synthesis of the new small polypeptides is shown in Table 1. In both experiments, wild-type phage was used at 30°. In experiment 1, a [^{14}C]aminoacid mixture was used as the label after phage infection. Three cultures of *E. coli* were infected with phage; these were labeled with ^{14}C at three different time intervals. None of the new polypeptides is labeled significantly in the 1- to 2-min interval and each is labeled at its highest rate in the 11- to 12-min interval. Polypeptide 3 appears to be labeled somewhat earlier than polypeptides 1 and 2. In experiment 2, [^{3}H]leucine was used as a preinfection label and was left in the media throughout the infection period. [^{14}C]Proline was used as the pulse label after infection. The minor polypeptide 4, but not 3, is labeled by the [^{14}C]proline (Fig. 1B). There is no significant incorporation of label into any of the polypeptides before 5 min after infection at 30°. Polypeptides 1, 2, and 4 are all labeled highest in the 8- to 11-min interval and there is still detectable incorporation into all three in the 18- to 21-min interval. Polypeptide 1, in particular, is labeled significantly in the 18- to 21-min interval, at 57% of its 8- to 11-min rate. The measurements of ^{3}H show that polypeptide 3 also is not labeled before 5 min after infection at 30°. The amount of ^{3}H in polypeptide 3 is as high at 11 min as at 21 min after infection. The amount of ^{3}H in the other polypeptides increases from 11 min to 21 min after infection, the increase being 130% for polypeptide 1, 50% for polypeptide 2, and 80% for polypeptide 4.

TABLE 1. *Kinetics of synthesis of the new polypeptides at 30°*

	Rate of ^{14}C-labeling* during intervals of:		
Exp. 1	1–2 min	5–6 min	11–12 min
Polypeptides			
1	<5	<5	13
2	<5	10	100
3	<5	46	83
Exp. 2	2–5 min	8–11 min	18–21 min
Polypeptides			
1	<2	13	7.5
2	<2	100	12
4	<2	29	6

In Exp. 1, three 200-ml cultures of *E. coli* were labeled with [^{3}H]leucine and then suspended in fresh M9 medium as described in Fig. 1A. At a cell concentration of 5×10^8/ml and at 30°, each culture was infected with T4D$^+$ phage at a multiplicity of 8. For each of the three time intervals shown, one culture was labeled by the addition of 0.1 mCi of a [^{14}C]aminoacid mixture containing 400 μg of each amino acid. The cultures were poured into an equal volume of crushed, frozen M9 medium and stirred rapidly. The cells were collected by centrifugation at 0° after several minutes.

In Exp. 2, three 200-ml cultures were labeled by addition of [^{3}H]leucine as in Fig. 1A. At a density of 5×10^8 cells/ml, the media were not changed. Rather, 100 ml of fresh M9 medium were added to dilute each culture to 3.3×10^8 cells/ml. After 10 min, T4D$^+$ phage, at a multiplicity of 8, was added to each culture. At the appropriate time, each culture was labeled with ^{14}C by addition of 0.1 mCi of [^{14}C]proline containing 160 μg of proline. At the end of each labeling period, chloramphenicol was added to give a final concentration of 50 μg/ml and the cultures were then poured into an equal volume of crushed, frozen medium. The cells were collected by centrifugation.

* The polypeptide with the highest label (^{14}C cpm) in each experiment was given an arbitrary value of 100.

Labeling experiments with maturation-defective amber mutants

Labeling experiments were performed with some of the maturation-defective amber mutants of phage T4, namely those in T4 genes 55 and 33 (8, 9). Fig. 3A shows the results of an experiment with T4 *am*BL292, a gene 55 mutant. The gel pattern of the enzyme from the experiment can be compared with that shown in Fig. 1A (T4 *am*N82 experiment), since the labeling conditions and gels used were similar. No significant amount of ^{14}C is present in the position of band 1. The small amount of ^{14}C in fractions 30 and 31 is in a contaminant that

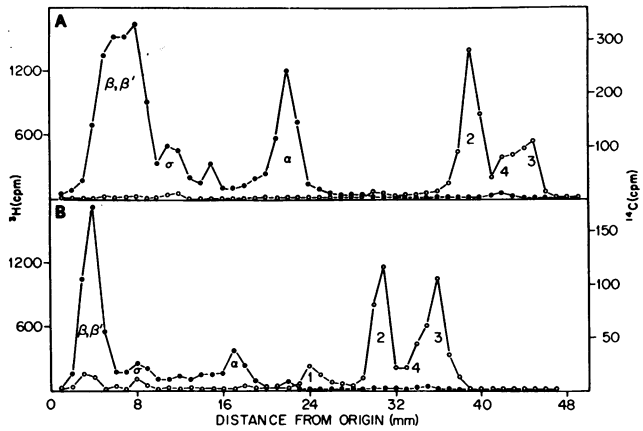

FIG. 3. Gel migration patterns of RNA polymerase from two labeling experiments with T4 *am*BL292 phage. The same labeling conditions were used for Fig. 3*A*, as for Fig. 1*A*. For infection, T4 *am*BL292 phage were added at a multiplicity of 8. For Fig. 3*B*, a 100-ml culture of *E. coli* CR63 was labeled with one-half the amounts of materials used in Fig. 3*A*. Otherwise, the conditions were similar, except that the labeling period was longer, from 1.5 to 22 min. The electrophoresis was performed in a 20% polyacrylamide gel rather than the usual 12%. ●——●, ^{3}H; O——O, ^{14}C.

moves slightly slower than band 1. T4 *am*552, another gene 55 mutant, was also used in a labeling experiment and its ^{14}C-labeling pattern was similar to that shown in Fig. 3*A*. When T4 *am*BL292 was used in an experiment with the permissive host, *E. coli* CR63, the labeling pattern of the enzyme was as shown in Fig. 3*B*. Since a 20% acrylamide gel was used, all the bands moved more slowly. ^{14}C-label is present in the position expected for polypeptide 1, although at a reduced level compared to that in Fig. 1*A* (T4 *am*N82).

Fig. 4 shows the results of two labeling experiments with T4 *am*N134, a gene 33 mutant. The experiment of Fig. 4*A* is similar to that of Figs. 1*A* and 3*A*. ^{14}C-Label is found in positions corresponding to bands 1, 2, and 3, but the shoulder on band 3 corresponding to that on band 4, is absent. Its absence was confirmed by use of [^{14}C]proline label after infection with T4 *am*N134 phage, so that band 4 could be detected directly (Fig. 4*B*). A comparison with the labeled enzyme isolated from cells infected with T4 *am*N82 (Fig. 1*B*) shows that the enzyme lacks most or all of polypeptide 4. Other gene 33 mutants have not yet been used.

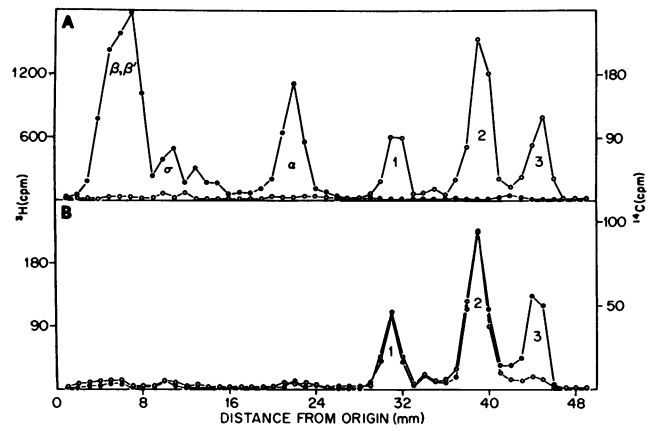

FIG. 4. Gel migration patterns of RNA polymerase from two labeling experiments with T4 *am*N134 phage. The same labeling conditions were used for Fig. 4*A* as for Fig. 1*A*. For Fig. 4*B*, the labeling conditions were the same as for Fig. 1*B*. ●——●, ^{3}H; O——O, ^{14}C.

Table 2 summarizes the results on labeled enzymes prepared from *E. coli* cells infected with wild-type T4 phage and with different early amber mutants. T4 DNA-negative amber mutants (8) in the early genes 44, 42 and 47, and 42 and 46 yielded results similar to those obtained with wild-type phage. The results obtained with the maturation-defective amber mutants in genes 33 and 55, already described above, are also summarized.

DISCUSSION

The presence of new small polypeptides bound to RNA polymerase of *E. coli* after infection of cells with T4 phage raises the old question: Do they really belong there? The results with the T4 maturation-defective mutants in genes 55 and 33 support the idea that the small polypeptides are functional new "subunits." Mutants in these two genes are unique among T4 mutants: After infection with such mutants, DNA synthesis takes place, but no late messenger RNA or protein is formed (8, 9). Bolle *et al.* (9) showed that there were two prerequisites for late transcription in T4-phage infected bacteria: (*a*) a competent form of DNA, requiring DNA synthesis after infection, and (*b*) the products of genes 55 and 33. Pulitzer (10), and Pulitzer and Geiduschek (11) showed that the gene 55 product is required continuously for late transcription, as shown in temperature-shift experiments with temperature-sensitive mutants in gene 55. The results presented here suggest strongly that a polypeptide with a molecular weight of 22,000 is the product of gene 55. The results obtained with a gene 33 mutant suggest that a polymerase-bound polypeptide with a molecular weight of 12,000 is the product of gene 33.

Small polypeptides belonging with the polymerase from cells infected with T4 phage were also detected by R. Horvitz and C. Goff* (personal communication) using another purification technique for the RNA polymerase. With polyacrylamide gel electrophoresis in the presence of SDS and urea, they have separated three polypeptides having molecular weights of 22,000, 15,000, and 12,000. Their enzyme apparently lacks the polypeptide designated polypeptide 3 in this paper. Horvitz has independently observed that the polypeptide with a molecular weight of 12,000 is absent when a gene 33 mutant is used, and he has confirmed my finding that the polypeptide with a molecular weight of 22,000 is absent when a gene 55 mutant is used.

The kinetics of synthesis of polypeptide 1 are in agreement with the *in vivo* findings of Pulitzer (10). He showed, using temperature-sensitive mutants in gene 55, that the product of gene 55 accumulates between 5 and 12 min after infection at 30°. By 12 min after infection, the amount needed to support late transcription at a maximal rate had been formed. Thus, RNA polymerase isolated from *E. coli* after 10 min of infection at 30 or 37° might be expected to catalyze the synthesis of RNA of the late messenger type (12). Using the σ subunit from uninfected RNA polymerase and T4 DNA from mature phage particles, others (5, 13, and 14) have found that enzyme

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TABLE 2. *Content of polypeptides in RNA polymerase from E. coli infected with different T4 amber mutants*

Phage	Gene	Host	Polypeptides 1 (% of total ^{14}C-label)	2 (% of total ^{14}C-label)	3 + 4 (% of total ^{14}C-label)
T4D$^+$	—	B	11	43	46
*am*N82	44	B	11	47	42
*am*N55-*am*B14	42, 46	B	10	45	45
*am*N55-*am*A456	42, 47	B	10	45	45
*am*N134	33	B	18	54	28
*am*BL292	55	B	<1	53	47
*am*552	55	B	<1	46	54
*am*N134	33	CR63	16	51	33
*am*BL292	55	CR63	5	45	50

All the labeling experiments were performed with a [^{14}C]-aminoacid mixture after infection, as described in Fig. 1*A*. The ^{14}C-label used in the calculations of the percentages above was that migrating faster than the α subunit of the polymerase on the gels.

from infected cells catalyzes the synthesis of only early messenger-type RNA (12). The failure of the enzyme to catalyze late messenger RNA synthesis may be due to failure of the *in vitro* systems to reproduce exactly one or more of the following *in vivo* factors: σ subunit; T4 template; reaction conditions such as pH and ionic strength. The σ subunit of the *E. coli* polymerase persists in the cells after T4 infection and is bound readily by normal core enzyme, as indicated by the labeling experiments reported here, but whether it is functional for late transcription is not known. The only *in vitro* preparations in which late T4 messenger RNA formation has been detected have been crude preparations of T4-infected cells (15). Snyder and Geiduschek (15) showed that late transcription by a crude DNA–RNA polymerase fraction from cells infected with T4 *am*BL292 was dependent on the addition of a supernatant fraction obtained from cells infected with T4 *am*N122, an early gene 42 mutant. The result suggested that the role of the gene 55 product was at the level of transcription.

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